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14. ABSTRACT
Prostate cancer (PC) cells have limited sensitivity to conventional therapeutic agents due to increased apoptotic thresholds but are highly sensitive to changes in their intracellular calcium levels. This distinction in apoptotic sensitivity may be exploited therapeutically but more information is needed to understand the respective mechanisms and processes involved. In our preliminary studies we identified two potential mediators of calcium-mediated apoptotic sensitization in PC cells. Calcium ionophore treatment of PC cells activated the calcium-sensitive protease calpain, stimulating two distinct pathways that regulate phosphotyrosine-initiated cell signaling (PTP1B) or directly trigger apoptosis (caspase 7). The role of caspase 7 and PTP1B in PC cell death and survival signaling was investigated using dominant negatives, siRNA and overexpression of these target proteins. Interference with caspase 7 activation did not appear to effect calcium-mediated cell death and induction of a calpain-proteolyzed variant of PTP1B (tPTP1B) had minimal impact on growth-factor or cytokine-mediated tyrosine phosphorylation or cell survival. Our results suggest that neither caspase 7 nor PTP1B play a major role in prostate cancer sensitivity to calcium-mediated apoptosis or survival signaling through tyrosine phosphorylation. However, due to impact on other kinases (Fak), tPTP1B may limit PC progression and metastatic behavior.

15. SUBJECT TERMS
Prostate Cancer, Apoptosis, Calcium

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INTRODUCTION

Prostate cancer (PC) cells have limited sensitivity to conventional therapeutic agents due to increased apoptotic thresholds but are highly sensitive to changes in their intracellular calcium levels. This distinction in apoptotic sensitivity may be exploited therapeutically but more information is needed to understand the respective mechanisms and processes involved. In our preliminary studies we identified two potential mediators of calcium-mediated apoptotic sensitization in PC cells. Calcium ionophore treatment of PC cells activated the calcium-sensitive protease calpain, stimulating two pathways that regulate phosphotyrosine-initiated cell signaling (PTP1B) or directly trigger apoptosis. The goal of this study is to determine the contribution of each of these processes to PC cell death and calcium flux sensitivity. The role of calpain as a master regulator of these processes is also to being investigated.

BODY

We have made great progress in completing the specific aims of this study. Listed below is an update on the progress with each aim.

Specific Aim 1. Examine the role of calpain in LnCaP cell death by inactivation of this enzyme with calpain inhibitors (chemical) or through overexpression of endogenous calpain inhibitors (calpastatin). Studies will be conducted on both wild-type and bcl-2 overexpressing LnCaP cells to determine the potential for bcl-2 to regulate calpain-mediated cell death (months 1-18).

Task 1 - measure apoptosis in calcium ionophore treated cells in the presence or absence of calpain inhibitors.

This aim has been modified since calpain inhibition by calpain inhibitors can induce apoptosis directly, possibly through effects on p53 stability. This activity complicates interpretation of the role for calpain in the activation of apoptosis or sensitization. To compensate for this unexpected toxicity two different approaches are being used to address the role of calpain in PC cell apoptosis. 1) Cells are treated with calcium ionophores in the presence or absence of calcium chelators before calpain activation and cleavage of PTP1B was monitored. Calcium chelators blocked both calpain activation, PTP1B proteolysis (activation) and its cytoplasmic translocation in LnCaP PC cells, demonstrating a direct role for calcium flux in activation of calpain and cytoplasmic translocation of PTP1B. To confirm a role for calpain in this process we are constructing an expression vector of anti-sense calpain-μ as well as protease-inactive (Cys to Ser Mutant) mutant of this gene. This construct is being subcloned into a doxycycline-sensitive expression vector (TRex). This vector has been used successfully to induce expression of other toxic or apoptotic gene products in LnCaP cells (see below). These new approaches are being used in conjunction with acute inhibitor studies to define the role of the calcium-calpain pathway in regulation of apoptotic activity in PC cells.

Although several approaches were tested, we were unable to fully assess the role of calpain in LnCaP cells in response to calcium flux. First we tried to overexpress μ-calpain in a tetracycline-inducible fashion using the TRex expression system. We obtained a μ-calpain expression plasmid from Melissa Spencer (Dept. of Neurology, UCLA) and subcloned it into the Bam H1 and Xba 1 sites of the pcDNA4/TO vector from Invitrogen's T-Rex system using gene specific primers. A cell-line optimization nucleofector kit was purchased from Amaxa Biosystems to optimize the electroporation of LnCaP cells and used according to the manufacturer's protocol. We determined that 70 % transfection efficiency was achieved by using 2 million cells in solution V at a setting of Q-27. Initially cells were electroporated with pcDNA6/TR and selected for stable expression of TetR using 5 μg/ml blasticidin. Individual foci were selected and expanded and tested for tetracycline-inducible gene expression by transient transfection with eGFP in the pcDNA4/TO vector. LnCaP clones that demonstrated highest eGFP expression determined by FACS scanning (BD FACS Vantage SE) in the presence (vs. absence) of 2 μg/ml tetracycline) were selected for subsequent electroporation of calpain in the pcDNA4/TO vector. Transfected cells were selected for zeocin (200 μg/ml) and blasticidin (5 μg/ml) resistance to obtain a stable cell line with inducible calpain expression. Twenty-four hours after tetracycline addition, cell lysates were assessed for calpain expression by immunoblotting. Unfortunately we saw no evidence of calpain induction following tet incubation, so additional mechanisms for evaluating calpain function in prostate cancer cell death were attempted.

Calpain siRNA was designed using the Ambion on-line program (Ambion Technical Bulletin #506). We synthesized 5 siRNAs and tested their silencing activity against endogenous μ-calpain 24, 48 and 72 hours after transfection. None of the siRNA sequences reduced calpain levels greater than ~ 25% and combinations resulted in

no more than 30% reduction. These experiments were costly and time consuming and we were unable to continue with calpain interference and over-expression studies.

Task 2 - compare in wild-type and bcl-2 overexpressing LnCap cells.

The studies described above were to be conducted on bcl-2 overexpressing PC cells. We proposed to analyze the effect of protease-inactive calpain-μ expression on bcl-2 overexpressing cells. However, modulation of calpain expression was not easily achieved by at least two separate techniques. Therefore this task was not completed due to difficulty in achieving calpain-activity deficient cells. We planned to obtain calpain deficient cells from knockout animals but at the time these animals did not exist and it was beyond the scope of this project to initiate this approach. There were also concerns regarding the use calpain-deficient fibroblasts or prostatic tissue from non-tumor bearing animals to investigate the role of calpains in calcium sensitivity in prostate cancer. Retroviral vectors carrying shRNA inserts to knockdown μ-calpain (pRetroSuper) are now available to complete this task in but resources were obligated toward completing other tasks.

Task 3 - transfect cells with calpastatin vectors and select stable clones expressing this protein. Measure calpain activity in calcium-ionophore treated cells, expressing calpastatin or transfected with empty vectors. Compare apoptotic sensitivity to challenge with calcium ionophores.

This aim has been modified to include tet-inducible expression of the calpastatin gene due to the toxicity of chronic calpain inhibition. We subsequently found that calpastatin was not an effective calpain inhibitor and did not continue to pursue this task.

Specific aim 2.

Examine the role of caspase 7 in calcium/calpain-mediated cell death by analysis of its in vitro activation with cell lysates derived from calpain activatable or inactivatable PC cells. The role of caspase 7 in calpain-stimulated LnCap cell death will also be assessed in vivo by measuring the effects of dominant-negative caspase 7 expression on calcium-mediated cells death (months 6-24).

Task 1 - measure caspase 7 proteolysis in calcium ionophore treated cells (in vivo). Compare in PC cells with activatable (LnCaP) and inactivatable (PC-3) calpains.

This aim was completed and we were unable to detect any significantly reproducible change in caspase 7 activation in calcium ionophore-treated LnCaP vs, PC-3 cells (data not shown). These results suggested that distinctions in calpain activation by calcium ionophore treatment were not associated with distinctions in caspase 7 activation. We conclude that calpains alone do not determine the caspase 7 response to calcium ionophores.

Task 2 - in vitro transcribe and translate caspase 7 and measure its proteolysis with calpain activated cell lysates. Compare with other caspases.

This aim has been completed as reported in an earlier progress report. Although calpain-mediated proteolysis of caspase 7 was detected it did not appear to be the major mechanism of caspase 7 activation in calcium ionophore treated PC cells.

Task 3 - transfect LnCaP cells with caspase 7 dominant negative vector. Select transfectants positive for DN caspase 7 expression (FLAG-tagged).

This aim is nearing completion. Dominant negative caspase 7 has been cloned and is active in partially blocking the effects of calcium ionophore on caspase 7 activation (see figure 1). However, we were unable to show overexpression of a wild-type caspase 7 or a caspase inactivated mutant (C186S) after transient transfection. After several attempts and two distinct expression systems, we were unable to obtain

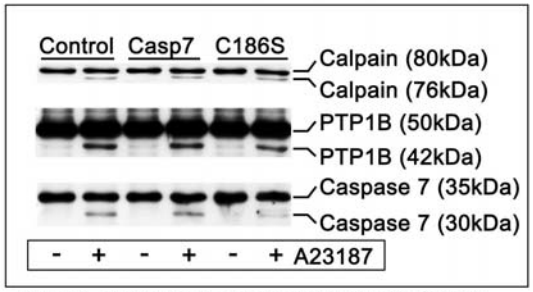

Figure 1. LnCaP cells were transiently transfected with pcDNA3 (empty vector - Control), pcDNA3-Caspase7 (Casp7) or pcDNA3-Caspase 7 mutant (C186S) and challenged with A23178 for 4 hours. Cell lysates were analyzed for calpain, PTP1B and Caspase 7 activation by immunoblotting. C186S caspase 7 mutant blocked Caspase 7 activation without affecting calpain or PTP1B cleavage. Stable transfectants are being selected to examine their effects on calcium ionophore mediated cell death.

stable caspase 7 of caspase 7DN mutant expressing PC cells.

Task 4 - measure cell death induced by calcium flux/calpain-activation in DN caspase 7 and vector control cells.

Although several techniques and expression vectors were explored we were unable to obtain stable DN-caspase 7 expressing clones. Therefore this experimental task was not successfully completed.

Specific aim 3.

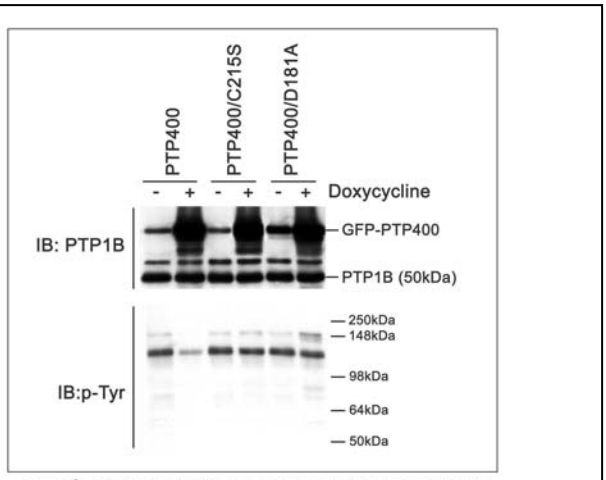

Figure 2. Effect of PTP400 expression on cellular p-Tyr levels in LnCaP cells. LnCaP cells were first transfected with the tetActivator and subsequently transfected with an vector that directs GFP-PTP400 expression vectors under the control of doxycycline. Stable transfectants were selected in the absence of tet. Several clones of each construct were screened for equivalent inducible expression of wild-type PTP400, a phosphatase-dead mutant (C215S) or a substrate-trap variant (D181A). Cells were left untreated or treated with doxycycline for 24 hours before cell lysates were screened for GFP-PTP expression and p-Tyr levels. Dox induced PTP400 expression of each construct. Only wild-type PTP400 expression affected p-Tyr levels in LnCaP cells (bottom).

Examine the effect of PTP1B expression on IGF-1 signaling and cell death in LnCaP and PC-3 cells using PTP1B expression vectors that encode a cytoplasmic form of PTPB (PTPB/400) that mimics the calpain-cleaved form of PTP1B (months 6-36).

Task 1 - transfect LnCaP and PC-3 cells with PTP1B/400. Select stable transfecants (FLAG-tagged).

Stable transfections could not be obtained by this approach due to the effects of expression of this gene on cell cycle and apoptosis of PC cells. We chose to express PTPB/400 under the control of a tet-activator (task 2). Much progress has been made in this aim.

Task 2 -if needed, subclone PTP1B/400 into a tetracycline-repressible vector (pEC1214). Transfect cells and select stable transfectants.

We were unable to obtain sufficient expression of PTP1B/400 using this vector. We chose to use the TRex tetracycline-activatible

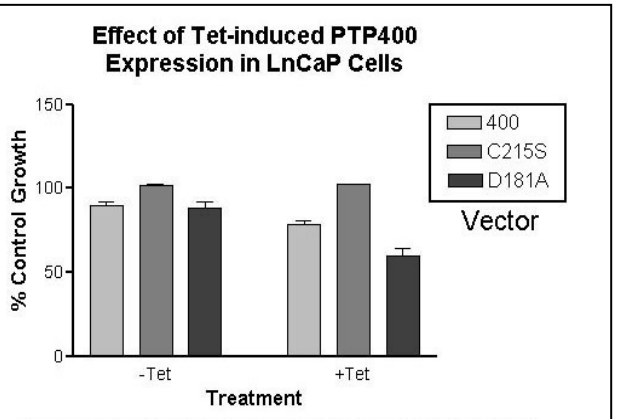

Figure 3. Effect of wild-type and variant PTP400 expression on the growth of LnCaP cells. LnCaP cells stabily transfected with PTP400 vectors (cloned downstream from a tet-activatable promoter- TRex expression system) were tet treated or left untreated for 72 hours before cell density was estimated by crystal violet staning. Wild-type PTP400 expression reduced LnCaP cell growth by ~ 20 %. Interestingly, the PTP substrate trap variant of PTP400 (D181A) had greater effects on cell growth (~ 40 % reduction) than w/t PTP400. Phosphatase-dead PTP400 (C215S) had no effect on cell growth.

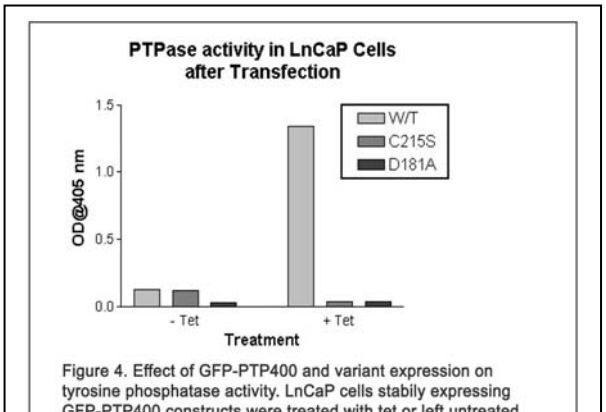

Figure 4. Effect of GFP-PTP400 and variant expression on tyrosine phosphatase activity. LnCaP cells stabily expressing GFP-PTP400 constructs were treated with tet or left untreated for 24 hours before cell lysates were subjected to IP wirh anti-GFP. Immunoprecipitates were washed and incubated with PTP substrate (para-nitrophenylphosphate) for 30 min before monitoring substrate hydrolysis as a measure of PTP activity (abs @405 nm). PTP activity was detected only with the w/t construct which was induced by ~ 8 fold with tet.

expression system and have obtained good results with this system (see figure 2). This construct was successfully used to monitor effects of PTP1B/400 on PC cell growth (figure 3) and PTP activity (figure 4).

Task 3 - measure effect of PTP1B expression on IGF-1 signaling by treatment with growth factor and monitoring IRS-1 phosphorylation, MAP kinase and Akt kinase activation. Compare empty vector, tetracycline-repressed and PTP1B/400 expressing transfectants.

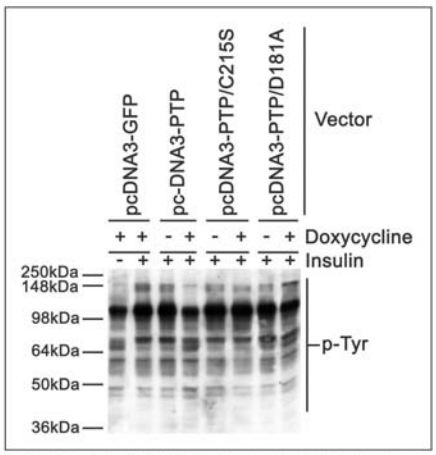

Figure 5. Effect of PTP induction on insulin-stimulated tyrosine phosphorylation. LnCaP cells stabily expressing PTP400 constructs (as noted) were treated with doxycycline for 24 hours or left untreated. Cells were treated with 10 µg/ml insulin (as noted) for 30 min before cell lysates were immunoblotted for phosphotyrosine. Only PTP400 reduced p-Tyr levels while PTP-D181A stabilized tyrosine phosphorylation of higher MW proteins.

This task was completed using PTP1B/400 expression under the control of the tet-activator. Induced cells were challenged with growth factors and substrate phosphorylation was defined by direct immunoblotting with phosphospecific antibodies. As shown in figure 5, PTP1B/400 expression reduced phosphorylation of a major tyrosine phosphoprotein (~120kDa) expressed in LnCaP cells that was activated by insulin. However, PTP1B/400 induction by doxycycline failed to suppress EGF-dependent tyrosine phosphorylation in LnCaP cells (figure 6), and PTP1B/400 induction had no consistent effect on activation or inhibition of MAPK which is known to be coupled to EGFR signaling and other receptor tyrosine kinase control. Although IL-6 failed to stimulate major changes in total cellular phosphotyrosine content (not shown), IL-6 mediated Stat3 activation was suppressed by wild-type and the D181A substrate-trap mutant of PTP1B/400 (figure 7). Interestingly, only the D181A variant of PTP1B/400 was able to suppress the growth of LnCaP cells suggesting that changes in turnover of phosphotyrosine substrates of PTP1B/400 were associated with Stat3 and growth inhibition (figure 3). This prompted an investigation of phosphoproteins associated with the substrate-trap variant of PTP1B/400.

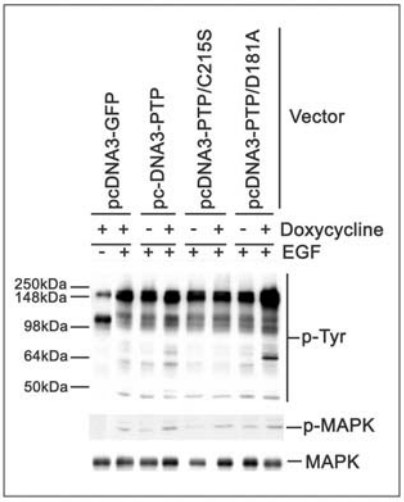

Figure 6. Effect of PTP induction on EGF-stimulated tyrosine phosphorylation. LnCaP cells stabily expressing PTP400 constructs (as noted) were treated with doxycycline for 24 hours or left untreated. Cells were treated with EGF (as noted) for 30 min before cell lysates were immunoblotted for phosphotyrosine. PTP400 induction had no affect on EGF-stimulated p-Tyr levels in LnCaP cells. However, PTP-D181A expression stabilized p-Tyr expression in LnCaP cells. These actions may correlate with the effects of PTPD181A on LnCaP cell growth (figure 4) and are currently being examined by immunoprecipitation and microsequencing.

We first sought to identify the 120kDa tyrosyl-phosphoprotein expressed in LnCaP cells that was suppressed by PTP1B/400 and secondarily to define the phosphoproteins associated with the PTP1B/400/D181A "substrate trap" mutant. Since previous reports described a role for focal adhesion kinase (Fak) and its homolog (Pyk2) in prostate cancer cell signaling, and metastasis LnCaP cells stabily expressing PTP400 and its mutants were screened for effects of tet-induction on tyrosine phosphorylation of Fak at autophosphorylation sites (Y576, Y577). As shown in figure 8, induction of w/t PTP400 resulted in a reduction in the recovery of pY-Fak but did not effect Fak protein levels. The phosphatase-dead PTP400 mutant (C215S) did not effect pY-Fak levels while induction of the substrate-trap mutant (D181A) resulted in minor reduction in pY-Fak levels. These results suggest that Fak is highly activated (tyrosine phosphorylated) in LnCaP cells and PTP400 reduces its level of activation. This PTP400-mediated inhibition may be associated with the small change in LnCaP cell growth (figure 3).

To confirm an association between PTP400 and pY-Fak, PTP400/D181A expressing LnCaP cells were tet induction and direct pY-Fak or GTP-immunoprecipitation. As shown in figure 9, pY-Fak was detectable in direct immunoprecipitates while irrelevant IgG failed to recover pY-Fak. Anti-GFP immunoprecipitates of GFP-PTP400/D181A (substrate-trap) resulted in recovery of GFP-PTP400 (bottom) and pY-Fak (top). Tetracycline induced PTP400/D181A expression and resulted in increased recovery of pY-Fak in anti-GFP immune complexes. Overall, the results support a role for PTP400 in regulation of pY-Fak levels in LnCaP cells. Additional studies in other prostate cancer cell lines are needed to determine the validity of this observation in regulation of prostate cancer cell signaling. Additional animal studies of PTP400 transfected LnCaP cells would be useful in determining the role of PTP400 in disease progression and metastases. However, these assays were not budgeted under this grant proposal.

To complement these studies and to provide a more rapid assessment of the role of PTP1B/400 on signaling, cell growth and apoptosis, we have completed construction of modified lentiviruses carrying the

PTP1B/400 gene. However, the lentivirus, including control viruses, induced toxicity in LnCaP and PC-3 cells, reducing their potential use for addressing the role of PTP400 in prostate cancer growth and signaling.

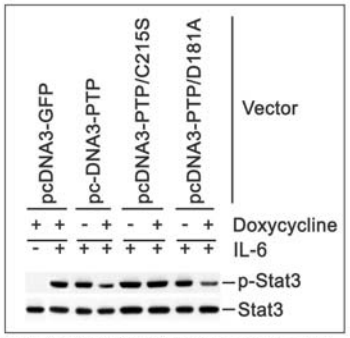

Figure 7. Effect of PTP400 expression on IL-6 mediated Stat3 activation. LnCaP cells stably expressing PTP400 constructs were treated with doxycycline (as described in figure 6) and challenged with IL-6 (10 ng/ml) for 30 min. Cell lysates were immunoblotted for phosphotyrosine-Stat3, stripped and reblotted for Stat3. Both w/t and D181A constructs of PTP400 reduced Stat3 phosphorylation.

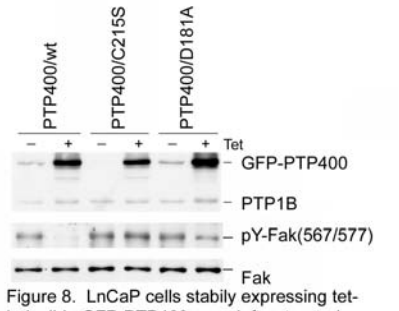

Figure 8. LnCaP cells stably expressing tet-inducible GFP-PTP400 were left untreated or treated with 2 μg/ml tet for 24 hours before lysates were collected and analyzed for PTP400 expression (top), pY-Fak (middle) and total Fak protein (bottom). PTP400 induction resulted in reduced tyrosine phosphorylation of Fak.

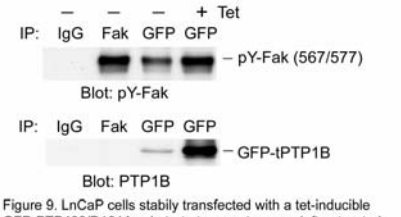

Figure 9. LnCaP cells stably transfected with a tet-inducible GFP-PTP400/D181A substrate trap vector were left untreated (-) or tet treated (+) for 24 h. Cell lysates were prepared and immunoprecipated with control IgG, anti-Fak or anti-GFP. The precipitates were resolved by SDS-PAGE and immunoblotted with anti-pY-Fak (top) or anti-PTP1B (bottom). The results demonstrate high level expression of activated Fak in LnCaP cells and affinity of PTP400/D181A for pY-Fak. Tet-induction of PTP400/D181A increased the recover of pY-Fak in GFP immunoprecipitates. These results suggest that pY-Fak is a substrate of the PTP400 form of PTP1B in LnCaP cells.

Task 4 - Measure apoptosis induced by calcium ionophore, cytokines (TNF, TRAIL) or radiation in transfectants expressing PTP1B/400 using standard apoptotic measurements.

Cell cycle changes and morphologic distinctions have already been completed. Apoptotic measures and variations in apoptotic sensitivity as well as other forms of cell death (anoikis) are under investiagtion. TRAIL receptor agonists currently in clinical trials for PC and other cancers (Human Genome Sciences) were proposed to be used in combination with PTP1B/400 lentiviral vectors to determine their potential to enhance cell death through activation of death receptors. Calcium ionophore and radiation-induced apoptosis will also be tested in combination with PTP1B/400 expressing cells. Unfortunately the limitations of the lentiviral expression vector were not anticipated and additional techniques are needed to complete this task.

KEY RESEARCH ACCOMPLISHMENTS

* Demonstrated that calcium flux was responsible to calpain activation and PTP1B proteolysis
* Established stably transfected LnCaP cells expressing the Tet activator (pcDNA6/TR transfectants; Invitrogen). We also established inducible expression of PTP400 (and variants). Caspase 7 dominant-negative and calpastatin were initially proposed to be studied in an inducible system. However, the limited impact of these proteins in preliminary studies diminished the need to pursue additional studies of these genes in PC.
* Determined that w/t PTP400 retained phosphatase activity.
* Induced PTP400 reduced insulin but not EGF stimulated tyrosine phosphorylation.
* Found PTP400 expression suppresses IL-6 mediated Stat3 activation.
* Determined that Fak is highly activated in LnCaP cells and PTP400 suppresses its tyrosine phosphorylation. Substrate trapping mutants of PTP400 also complex with pY-Fak in PC cells.
*Have constructed lentiviral vectors for PTP400 cloned downstream of an IRES site and GFP expression cassette. Found lentiviral toxicity in PC cells.

REPORTABLE OUTCOMES

ted several cell lines, constructs and vectors useful for direct assessment of the genes of interest to this project. The cell lines and vectors will also be made available to other investigators. We have not yet completed these studies but are poised to complete this project. Most of the studies were completed in one PC cell line and additional studies are needed to confirm our observations of PTP400 activity and target specific effects in PC cells.

One student has been granted her doctorate degree (Sharon Beresford) as a direct consequence of her involvement with this project. A technician currently working on this project has decided to apply for and M.B.A. so that he can pursue a career in the biotechnology field (Jonathan Stapley). The preliminary data on PC cells looks very promising and an NIH grant to advance this work and conduct animal studies of metastatic PC will be submitted upon completion and publication of this preliminary work.

CONCLUSIONS

The role of two calcium sensitive pathways in PC cell death is under investigation. These studies are focused on defining the role of calpain-sensitive protein in this process. The difficulty exists due to the nature of the proteins under investigation since expression of both PTP1B and caspase 7 are directly detrimental to PC cells. To understand their involvement in PC cell death these proteins are being expressed or inactivated by overexpression of a dominant negative variant or tet-inducible construct. Thus far our data clearly show that calcium flux (not calcium ionophore itself) induces calpain activation and PTP1B proteolysis resulting in its cytoplasmic translocation. Expression of a dominant negative caspase 7 has limited effect on endogenous caspase 7 activation by calcium ionophore. To further understand the role of PTP1B in PC cell death and signaling, a cytoplasmic form of the PTP1B protein was under the control of a tetracycline promoter (GFP-PTP400). Our results demonstrate that specific signaling pathways (i.e. insulin, IL-6) are inhibited by the cytoplasmic form of PTP1B (PTP400). Signal inhibition has little effect on LnCaP cell growth and responses in cells challenged with apoptotic stimuli have yet to be determined. We have been successful in generating a lentiviral vector to expedite PTP1B studies. However, we were unable to advance this work due to the unforeseen toxicity of control lentiviral vectors, particularly in PC-3 androgen independent cells. However, inducible expression of PTP400 has demonstrated that IL-6 and insulin signaling cascades are inhibited in LnCaP cells. In addition we determined that PTP400 suppresses Fak phosphorylation and due to the role of Fak in metastatic disease anticipate impact of PTP400 in the progression and metastatic behavior of LnCaP tumors. These studies are of significance since PC lethality is predominantly associated with distal site metastases and therapeutic options for advanced PC tumors are limited. Overall, our goals of defining a role for PTP400 in PC cell signaling and growth inhibition have being completed through these studies. Our future goal will be to advance our basic science observations into new therapeutic agents or strategies to treat PC patients.

REFERENCES

N/A

APPENDICES

N/A